Histopathological and Inflammatory Features of Chronically Discharging Open Mastoid Cavities Secondary Analysis of a Randomized Clinical Trial

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IMPORTANCE Many patients with an open radical mastoid cavity experience therapy-resistant otorrhea. Little is known about the underlying histopathological substrate of unstable cavities and the correlation with treatment failure.

OBJECTIVE To study the histopathological and inflammatory features of chronically discharging open radical mastoid cavities and the influence of different treatments.

DESIGN, SETTING, AND PARTICIPANTS This secondary analysis of a randomized clinical trial was a histopathology study of tissue samples of a cohort of 30 patients with a chronically discharging open mastoid cavity. Samples were taken from the cavities, which were treated with either honey gel or conventional eardrops in a tertiary center between 2012 and 2013. Tissue staining was performed in May 2014; final computer analysis/correlation studies were performed in June 2016.

MAIN OUTCOMES AND MEASURES Differences of epithelial tissue coverage, infiltration of T cells (CD3, CD4, CD8) and macrophage (CD68, isoenzyme nitric oxide synthase, arginase 1) (sub-)populations, infection status, and the correlation with clinical presentation.

RESULTS There were 30 patients (24 [80%] male; mean [SD] age, 59 [14] years). Cavities were covered with either stratified squamous (keratinized) epithelium (n = 10), respiratory columnar epithelium (n = 9), or granulation tissue (n = 10). The presence of respiratory epithelium was associated with lower treatment success (posttreatment VAS improvement of 3.1 [95% CI, 0.5 to 5.8] for discomfort and 3.6 [95% CI, 0.2 to 6.9] for otorrhea in the group with granulation tissue coverage vs 4.9 [95% CI, 0.2 to 9.6] and 5.8 [95% CI, −0.1 to 11.6] in the group with squamous [keratinized] epithelium coverage and 1.4 [95% CI, −1.2 to 4.1] and 2.5 [95% CI, −1.3 to 6.2] in the group with respiratory columnar epithelium coverage). In all 3 tissue types of cavity-covering tissues, T-cell infiltrates consisted of helper T cells and cytotoxic T cells, together with a lower number of macrophages. The immunopositivity for isoenzyme nitric oxide synthase and arginase 1 was high and not restricted to a macrophage subpopulation, but seen in various cell types. Inflammatory infiltrations varied strongly in all 3 tissue modalities.

CONCLUSIONS AND RELEVANCE Discharging open mastoid cavities can be classified histologically into 3 different types, based on their coverage: squamous epithelium, respiratory epithelium, or granulation tissue. Treatment is less successful in cavities covered with respiratory epithelium, possibly explained by the status of bacterial infection and local immunological differences.
Canal-wall-down mastoid surgery is a common procedure to manage cholesteatoma and chronic otitis media. Despite a low rate of recurrence and satisfactory results, more than 20% of patients with an open mastoid cavity experience intermittent or continuous otorrhea, which is often resistant to therapy. It seems that a major problem of unstable cavities is insufficient (re)epithelialization, which is favored by local conditions, unfavorable cavity shape, and size and host factors. Recently we presented promising results with the topical treatment of chronically discharging open mastoid cavities with honey gel, which led to less discomfort, otorrhea, inflammation, and infection than conventional eardrops. In other wounds, it was already shown that honey treatment stimulates wound healing, wound debridement, and epithelialization. Chronically discharging cavities resemble chronic wounds by remaining in an uncoordinated, self-sustaining state of inflammation. In these wounds, an abundance of proinflammatory macrophages seems to hamper wound progression and healing. This type of macrophage outbalances wound-healing macrophages and is stimulated by T-helper type 1 cells. Honey has an immune-modulatory effect and could contribute to a better wound healing on this cellular level.

Despite different treatment approaches, little is known about the underlying histopathological substrate of unstable cavities. Therefore, in this study we present the histological results of biopsies taken before and after treatment, during a 12-week clinical study, in which chronically discharging open radical mastoid cavities were treated with either medical honey or conventional eardrops, as published elsewhere. We aimed to investigate the histopathological features of unstable cavities and their correlation with clinical presentation and treatment response, with a special focus on proinflammatory immune mechanisms by T-cell and macrophage subsets.

Methods

Study Population

Histological samples were obtained from 30 patients who were enrolled in a clinical study as previously described. The study was approved by the ethics committee of Maastricht University Medical Center and all patients gave their written informed consent prior to the start of the study. Briefly, patients with a chronically infected open mastoid cavity were recruited from April 2012 until September 2013 in a single-center, randomized controlled, double-dose trial, conducted at the Maastricht University Medical Center, The Netherlands. After inclusion, a swab sample and a biopsy were taken from the cavity and patients were treated with either Terra-Cortril Polymyxin B eardrops (hydrocortisone, oxytetraacycline, and polymyxin B) for 1 week or the medical honey gel NasuMel (Revamil honey mixed with water and hydroxyethylcellulose). Treatment was repeated after 4 weeks. A second swab sample and biopsy were taken 8 weeks after the start, and patients filled in a visual analog scale (VAS) about their cavity problems.

Biopsies

The site of biopsy was topically anesthetized with lidocaine, 10%, for 10 minutes, and a small tissue sample of several millimeters was taken with a Blakesley forceps. The biopsy was taken from the part of the cavity with macroscopically most signs of infection, that is, granulation tissue, pus, or erythema. When no signs of infections were present, a random biopsy sample was taken.

Immunohistochemical Staining and Evaluation

Tissue specimens were immediately fixed in 4% buffered formaldehyde and processed by regular histological procedures. Biopsies were paraffin embedded and sectioned in 4-μm slices. Parallel sections were stained with hematoxylin–eosin and periodic acid–Schiff stains. Tissue sections were scored for extent of epithelial tissue coverage and presence of inflammation by 2 independent observers (C.J.P.-K. and D.H.). Tissue was obtained from the Maastricht Pathology Tissue Collection. Collection, storage, and use of tissue and patient data were performed in agreement with the Code for Proper Secondary Use of Human Tissue in the Netherlands (https://www.federa.org). Sections were also immunohistochemically stained with monoclonal antibodies defining T cells and macrophages: CD3 (total T cells), polyclonal rabbit anti-human-CD3 (Dako); CD4 (helper T cells), monoclonal mouse anti-human-CD4 (Dako); CD68 (macrophages), monoclonal mouse anti-human-CD68 (Dako); and isoenzyme nitric oxide synthase (iNOS) and arginase 1 (Arg-1) (wound healing markers in macrophage subsets), polyclonal rabbit anti-human-iNOS antibody (Abcam) and polyclonal rabbit anti-human-Arg-1 (provided by P. van Dijk, Maastricht University, Netherlands), respectively. Computer-assisted color imaging analysis was performed using the histomorphometry software Leica Qwin, version 3. Macrophage and T-cell content was expressed as percentage of positive cells of total tissue area.

Statistical Analysis

Normally distributed continuous data were compared using the paired t test and effect size was expressed using the Cohen d. The exact binomial test in the R software package was used to calculate the probability of a positive test result of microbiological swabs. Mean difference data were reported, including a 95% CI.

Key Points

Question What is the histopathological origin of unstable open mastoid cavities and its correlation with treatment failure?

Findings This study of tissue samples from a cohort of patients in a randomized clinical trial with chronically discharging open radical mastoid cavities found that histologically, 3 different types of unstable open mastoid cavities can be discriminated on the basis of their coverage: stratified squamous (keratinized) epithelium, respiratory columnar epithelium, or granulation tissue. Treatment of patients with respiratory epithelium coverage is less successful, likely as a result of bacterial infection.

Meaning In patients with chronically discharging open mastoid cavities, typing of the cavity coverage can be important for treatment expectations.
Results

Clinical Outcome

A previously reported cohort of 30 patients (24 male) with a mean (SD) age of 59 (14) years was used for this study. Patients had an open cavity for a mean of 19 years (range, 1-57 years), and 8 (27%) had continuous problems with it since they underwent surgery. Eighteen were treated with honey gel and 12 with eardrops. Topical honey treatment led to less discomfort and otorrhea (measured by VAS) and a macroscopically detectable improvement of cavity inflammation, compared with eardrops. Of all cavities, 8 (27%) were colonized with *Pseudomonas* species, 6 (20%) with *Staphylococcus aureus*, and 7 (23%) with other species. The incidence of pathological bacterial infection was reduced by treatment for 4 (22%) in the honey group, compared with 3 (30%) in the eardrop group.

Histopathological Analysis of the Chronically Discharging Open Mastoid Cavity Shows Different Subtypes

Thirty biopsies were taken before patients were treated with either honey gel or eardrops. Twenty-nine tissue fragments were available for histological analysis. Three different types of epithelial tissue coverage were found in the biopsies: coverage with either stratified squamous (keratinized) epithelium, respiratory (ciliated) columnar epithelium, or granulation tissue without epithelial coverage. In 10 patients (34%), tissue fragments were covered with stratified squamous (keratinized) epithelium, in some with hyperkeratosis. In all these biopsies, inflammation was present, with mainly lymphoplasmacellular and plasmacytoid infiltration with neutrophils. In some samples, eosinophils and macrophages were seen as well.

In 6 patients (21%), tissue was additionally focally lined with (pseudo-stratified) ciliated columnar epithelium, which was associated with more extensive inflammatory infiltration including lymphocytes, plasma cells, and neutrophils. In 3 tissue fragments (10%), only ciliated columnar epithelium was present, with in subepithelial layers fibrosis with prominent active, chronic inflammatory infiltration, with lymphocytes, polymorphonuclear neutrophils, macrophages, and some eosinophilic granulocytes.

Ten tissue fragments (34%) of cavity coverage consisted of granulation tissue with different degrees of fibrosis and active, chronic inflammatory infiltration with lymphocytes, eosinophils, polymorphonuclear neutrophils, plasma cells, and occasionally macrophages and giant cells. Inflammation was accompanied with vascular proliferation in most samples. One tissue sample was damaged and could not be analyzed.

Histological Features Correlated With Reported Clinical Outcome

Patients filled in a VAS about discomfort and otorrhea before the treatment and 4 weeks after the second treatment (8 weeks later), when the second biopsy was taken. The histological differences of an unstable cavity seemed to be associated with the outcome of successful treatment. Patients with a cavity with a (partial) coverage of granulation tissue or with a coverage of stratified squamous (keratinized) epithelium prior to treatment showed a much better VAS improvement (treatment response), compared with patients with a cavity (partially) covered with respiratory columnar epithelium. This response was irrespective of the treatment modality. The improvement of VAS after treatment was 3.1 (95% CI, 0.5 to 5.8) for discomfort and 3.6 (95% CI, 0.2 to 6.9) for otorrhea in the group with granulation tissue coverage prior to treatment, compared with, respectively, 4.9 (95% CI, 0.2 to 9.6) and 5.8 (95% CI, −0.1 to 11.6) in the group with squamous (keratinized) epithelium coverage and 1.4 (95% CI, 1.2 to 4.1) and 2.5 (95% CI, −1.3 to 6.2) in the group with respiratory columnar epithelium coverage.

In addition, there was a nonsignificant finding that a microbiological swab sample that was positive for pathologic bacteria was possibly associated with a cavity (partially) covered with respiratory epithelium. In patients with (partial) coverage with granulation tissue, a swab sample positive for pathologic bacteria was found in 50% (n = 5; 95% CI, 19%-81%), compared with 60% (n = 6; 95% CI, 26%-

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**Figure 1. Photomicrographs of 3 Different Tissue Specimens Obtained Prior to Treatment**

A Stratified (keratinized) epithelium coverage

B Granulation tissue coverage

C Ciliated columnar epithelium coverage
88%) in the group with squamous (keratinized) epithelium coverage and 78% (n = 7; 95% CI, 40%-97%) in the group of patients with (partial) coverage with respiratory columnar epithelium. However, no association was seen with gram-positive or gram-negative species.

The (partial) cavity coverage changed considerably after treatment. In 10 patients, biopsies showed granulation tissue coverage before treatment. After treatment in this group 4 biopsies changed to squamous (keratinized) epithelium coverage. In the group in which biopsies consisted of respiratory columnar epithelium coverage (10 patients), this coverage changed to squamous (keratinized) epithelium in 7 patients. In patients who had coverage with squamous (keratinized) epithelium prior to treatment (10 patients), this coverage stayed unchanged in 6 patients and changed to coverage consisting of granulation tissue in 2 patients (2 patients of this group were excluded). Thus, regardless of treatment, a high proportion of granulation and respiratory epithelium coverage altered to coverage with squamous epithelium.

Immunohistochemical Features of the Chronically Discharging Open Mastoid Cavity

T-Cell and Macrophage Infiltration

In all 3 epithelial tissue modalities, varying intensities of CD3-positive T cells were present, mainly in underlying granulation tissue. Subtyping of this population showed that helper T cells (CD4 positive) and cytotoxic T cells (CD8 positive) were present in equal numbers within the biopsies. We found no skewing of the immune response and no specific association of T-cell phenotype within either of the histological subtypes (Figure 3). Furthermore, we found relatively low numbers of macrophages (CD68 positive) in the biopsies (Figure 3). The staining of CD68-positive macrophages was not associated with histological subtypes, although there seemed to be a higher abundance of macrophages in areas of fibrosis.

INOS and Arg-1 Positivity

A constitutive iNOS immunopositivity was seen diffusely and intensively in squamous and respiratory epithelium, as well as a moderate to low intensity for endothelial cells, smooth muscle cells, and fibroblasts. A moderate to strong positivity was seen for macrophages, without specificity for a macrophage subpopulation and other immune cells. Overall, Arg-1 expression followed a similar pattern and almost seemed to be co-expressed with iNOS in epithelial cells, inflammatory cells, and mesenchymal cells (eFigure in the Supplement). Here as well, no specific macrophage subtype (proinflammatory or wound healing) was identifiable. Because of the strong expression pattern of both iNOS and Arg-1, we could neither quantitatively discern differences in expression levels between histological subtypes nor between treatment groups.

Discussion

In this histopathological study, we show that chronically discharging open mastoid cavities are (partially) covered with 3 different types of tissue, either stratified squamous (keratinized) epithelium, respiratory (ciliated) columnar epithelium, or granulation tissue without an epithelial coverage. Treatment of patients with respiratory epithelium coverage seemed to be less successful, likely as a result of bacterial infection. All 3 types of biopsies with different epithelial coverage show T-cell infiltration, consisting of more or less equal numbers of helper and cytotoxic T cells. Also, low numbers of macrophages were present in the 3 tissue types. The 3 types of tissue coverage show a high immunopositivity for iNOS and Arg-1 in almost all macrophages. Therefore, no indication of macrophage skewing toward a proinflammatory or anti-inflammatory (wound healing) phenotype of the immune response was detected.
Until now, little has been known about the underlying histopathological features of an unstable, chronically discharging radical mastoid cavity. The postoperative and post-inflammator healing of an open cavity can only partly be compared with wound healing elsewhere. In a cavity, skin has to grow on bare bone, a difficult process, which is further hindered by a humid environment, exudation, infection, and keratin debris. Normal wound healing consists of a well-structured process of inflammation, proliferation, and remodeling, in which reepithelialization is essential for wound closure. The latter is closely associated with granulation tissue formation. The moist environment of an open cavity favors granulation tissue growth, which again interferes with epithelialization.

We show in this study that the coverage of an unstable open cavity consists of 3 different tissue types, that is, stratified squamous (keratinized) epithelium, respiratory columnar epithelium, and granulation tissue. Importantly, this histological classification at the moment of clinical interference is associated with treatment success. A possible explanation for this finding is that granulation tissue formation and reepithelialization are spatially and temporally closely correlated in wound healing and a better treatment response is reasonable. We hypothesize that respiratory epithelium in contrast followed a process of metaplasia, rather than normal epithelial differentiation.

Honey and conventional eardrops were both effective in treating unstable cavities, as shown earlier. The coverage of unstable cavities changed profoundly as well. Most cavities that had been covered with granulation tissue prior to treatment changed to coverage with squamous epithelium after treatment. This was regardless of the treatment modality. We noticed baseline differences in the different sorts of cavity coverage, with more cavities covered with granulation tissue in the honey group, which was also seen after treatment. Possible explanations for histological differences that were seen in our study are the influence of chronic bacterial infection and local immune reactions. The presence of endotoxins, cell wall products from gram-negative bacteria, has been shown to lead to a treatment-dependent switch in subtype populations could be responsible for wound healing progression. Contrary to these expectations, we observed a high immunopositivity for both iNOS and Arg-1 in various cell types, without specificity for macrophages. Other studies showed a high expression for iNOS in epithelial, endothelial, and smooth muscle cells, as well as macrophages, fibroblasts, and polymorphonuclear neutrophils in wounds. In infected chronic wounds, increased arg-1 levels are also found. Different sources for this enhanced expression are reported, as polymorphonuclear neutrophils, wound margin keratinocytes, fibroblasts, and macrophages. An altered high iNOS and Arg1 expression is associated with chronic wound healing conditions. The enhanced expression of both iNOS and Arg-1 in nonhealing mastoid cavities indicates an important role of NO metabolism in this chronic healing problem. This is because different medications are interacting with the NO metabolism during wound healing. As discussed earlier, honey seems to be an effective treatment of unstable cavities. It was shown in different models that honey has a positive influence on NO metabolism, with a net anti-inflammatory effect. In contrast, there are indications that corticosteroid application has a negative effect on NO metabolism in the skin, and during inflammation. This knowledge can influence future treatment strategies. In the Netherlands, common treatments of chronically discharging cavities are repeated debridement, gentian violet application, local cautery, and boric acid powder treatment, which has a good proven effect in otitis.

On the basis of the results of this study, it is not possible to advise specific treatment, but we can argue that coverage of a mastoid cavity with respiratory columnar epithelium is associated with treatment failure and possibly bacterial infection and necessitates a long-lasting and more repetitive treatment strategy or even revision surgery. Furthermore, novel drugs, such as NO-delivering medications that have a positive effect on wound healing, could play an important role in the disturbed NO metabolism in the nonhealing cavity.

Limitations
To our knowledge, this is the first study that shows the histological differences of cavity coverage and the underlying immunological substrate in patients with an active discharging open mastoid cavity. This study is in line with earlier post-mortem studies, which also showed that unstable cavities were predominantly covered with stratified squamous keratinized epithelium with fibrosis and inflammatory infiltrate in subepithelial layers. There, coverage with respiratory epithelium was accompanied with a more intense inflammatory infiltrate.
Conclusions

In patients with chronically discharging open mastoid cavities, typing of the cavity coverage is important for treatment expectations. Histologically, 3 different types of coverage can be discriminated, either stratified squamous (keratinized) epithelium, respiratory columnar epithelium, or granulation tissue. Respiratory columnar epithelium is associated with treatment failure and with bacterial infection.


